Amendments to the Specification:

Please replace the first paragraph on page 2, lines 7-11 with the following amended paragraph:

This application is a continuation of co-pending U.S. Application Serial No. 09/549,926 filed April 14, 2000, which is a continuation-in-part of the following applications: U.S. Application Serial No. 09/120,264, filed July 21, 1998, which is a continuation-in-part of U.S. Application Serial No. 09/087,210, filed May 28, 1998, which is a continuation-in-part of U.S. Application Serial No. 08/864,357, filed May 28, 1997. The disclosures of each of the aforementioned applications are incorporated herein by reference.

Please replace the first paragraph on page 3 lines 9-15 with the following amended paragraph:

Amino acid analysis of purified human uteroglobin reveals that it is structurally similar but not identical to other uteroglobin-like proteins, e.g. rabbit uteroglobin; 39 of 70 amino acids are identical between human and rabbit uteroglobin. (see Figure 21). The uteroglobin-like proteins, including human uteroglobin/CC10, rat uteroglobin, mouse uteroglobin, and rabbit uteroglobin, exhibit species-specific and tissue-specific antigenic differences, as well as differences in their tissue distribution and biochemical activities *in vitro*. Uteroglobin-like proteins have been described in many different contexts with regard to tissue and species of origin, including rat lung, human urine, sputum, blood components, rabbit uterus, rat and human prostate, and human lung.

Please add the following paragraphs at page 12 line 16:

Figure 21 shows an alignment of UG-like proteins; (Human CC10 (SEQ ID NO: 1), Rabbit UG (SEQ ID NO: 2), Rat CC10 (SEQ ID NO: 3), and Mouse CC10 (SEQ ID NO: 4))

Figures 22A, 22B, and 22C show the construction of a new gene for rhUG and its cloning into expression vector pPL-lambda.

Please add the following paragraphs at page 21 line 15:

Construction of Bacterial Gene for rhUG

A synthetic gene for human UG is assembled from oligonucleotides and cloned into pBR322 for DNA sequencing to confirm the sequence. Because native UG has a glutamic acid residue at its N-terminus, an initiator methionine will be added. Mantile et al (1993; supra) have previously shown that addition of Met-Ala-Ala at the N-terminus of recombinant hUG does not alter its activity as an inhibitor of PLA.sub.2. Nor does a Met residue at the N-terminus (which allows bacterial translational initiation) effect is activity as a PCB-binding protein (Hard et al, 1996; supra). Bam H1 sticky ends are located at both ends of the annealed gene to facilitate cloning into the Bam H1 site of pBR322, which allows identification of clones by screening for sensitivity to tetracycline. Codon usage will be optimized for expression in bacteria according to Anderssen and Kurland (Codon Preferences in Free-living Microorganisms. Microbiological Reviews. 54:198-210 (1990)). Thus far, it has only been expressed from the human cDNA sequence with only moderate levels of protein expression and recovery achieved. The optimization of codon usage typically results in a higher translation efficiency and expression level. Oligos 1-4 represent the coding strand and 5-8 represent the complementary strand. Both sets are in order from 5' to 3', respectively, and are assembled as in FIG. 2.

Table of Oligonucleotides

5'-GATCCATGGAAATCTGCCCGTCTTTCCAGCGTGTTATCGAAAC
CCTGCTGATGGACACCCCGTCC-3' (SEQ ID NO:5)

5'-AGCTACGAAGCAGCTATGGAACTGTTCTCTCCGGACCAGGA CATGCGTGAA GCAGGTGCT-3' (SEQ ID NO:6)

5'-CAGCTGAAGAAACTGGTTGACACCCTGCCGCAGAAACCGCGTG AATCCATCATAAACTG-3' (SEQ ID NO:7)

5'-ATGGAGAAGATCGCTCAGTCTAGCCTGTGCAACTAAG-3' (SEQ ID NO:8)

5'-CTTAGTTGCACAGGCTAGACTGAGCGATCTTCTCCATCAGTTTG ATGATGGATTCACGCG-3' (SEQ ID NO:9)

5'-GTTTCTGCGGCAGGGTGTCAACCAGTTTCTTCAGCTGAGCACT GCTTCACGCATGTCCT-3' (SEQ ID NO:10)

5'-GGTCCGGAGAGAACAGTTCCATAGCTGCTTCGTAGCTGGACG GGGTGTCCATCAGCAGGG-3' (SEQ ID NO:11)

5'-TTTCGATAACACGCTGGAAAGACGGGCAGATTITCCATGGATC-3' (SEQ ID NO:12)

Subcloning of Coding Sequence into Expression Vector

Oligonucleotides homologous to the 3' and 5' ends of the new gene are then used to amplify the gene from pBR322 by PCR and clone it into pP.sub.L -lambda. The cloning strategy,

as well as a map of the expression vector is shown in FIG. 2. Restriction sites are incorporated into the ends of the flanking oligonucleotides to facilitate directional cloning of the gene into the vector.

Ligation mixtures are transformed into the first strain (NM4830-1 for pP.sub.L -lambda) and plasmid-bearing colonies are selected with ampicilin. Transformants are initially screened with a quick PCR assay done on the bacterial colonies to determine insert size. The secondary screen is done in 10 ml bacterial cultures for expression of an induced protein of the appropriate size (10 kDa). Samples of whole cells, induced for expression of the uteroglobin, directly lysed in 2.times. gel loading buffer, are run on 16% Tris-glycine SDS-PAGE gels in a minigel apparatus. Plasmid DNA from clones that exhibit overexpressed induced UG is then prepared and the DNA sequence is verified. Both plasmid DNA and bacterial strains from positive clones are then frozen down and stored. Clones for expression are maintained on LB plates containing ampicillin. These are streaked weekly for up to 10 passages, after which a fresh streak is taken from a frozen seed vial for serial culture, to insure strain authenticity.

Bacterial Host Strain Construction

An E. coli host strain is constructed for expression of rhUG from the heat-inducible promoter, P.sub.L, from a bacteriophage lambda. This promoter requires the lambda repressor, cI, in order to remain inactive until the desired time of expression. The temperature sensitive cI protein (mutant cI.sub.453) binds to the P.sub.L promoter when the temperature is 32 degree C. or less (Sambrook, et al, 1989). When the temperature is rapidly elevated to 42.degree. C. (i.e., less than 15.degree.), the cI.sub.453 repressor undergoes a conformational switch and no longer binds the P.sub.L promoter sequence. This results in overexpression of the rhUG gene. The bacterial host strain is derived from wild type E. coli, strain W3110, obtained from the ATCC.

Wild type strains are generally regarded as more robust than typical research cloning strains and are preferred for protein production. A lysogeen of this strain is constructed using a cI.sub.453, Xis- mutant of lamba phage, also obtained from the ATCC. This strain constitutively expresses the cI.sub.453 repressor. Since the lysogen is Xis-, the prophage cannot excise itself from the host chromosome, preventing replication of the phage when expression of the protein is induced. This host strain, W3110.lambda.cI.sub.453, is transformed with the expression vector and maintained as described previously. Individual colony transformants are also screened for expression as described.

Expression of rhUG

Clones selected for expression of rhUG are inoculated from colonies on solid media into 50 mL of broth and shaken overnight. This starter culture is used to inoculate 250 ml rich media containing ampicillin at 100 micrograms per mL in shaker flasks. These cultures are grown at 32.degree. C. until they reach an optical density of 0.5 at 600 nm. Expression of rhUG is then induced with a heat shift to 42.degree. C. The culture is shaken for an additional 2-4 hours at 42.degree. C. The cells are then harvested by centrifugation, washed once with PBS, and stored at -20.degree. C. as a frozen cell pellet until analyzed. These cells pellets are used in the initial stages of the development of a purification process.

Storage and Stability of Expression Strains

Up to four strains that overproduce large quantities of active rhUG are cultured, without expression. Culture conditions for preparation of seed stock are slightly different from those used in protein expression with the same strains, due to the different objectives for growing the cultures. Seed stock cultures are grown under conditions that minimize expression and enhance stability, ie. the temperature is kept below 30 degree C. and media is minimal with appropriate

supplements. Seed stock material is grown to early stationary phase in ten liter batches in Microferm fermenters (New Brunswick), gently pelleted and frozen down (-70 degree C.) in 4% glycerol in aliquots equivalent to 200 mL of culture. Likewise, lyophilized material is generated from several liters worth of bacterial culture for storage. Stored bacteria are evaluated for stability of plasmid, DNA sequence and expression level at six month intervals.

Fermentation Optimization

Of the four expression strains selected for storage, the best two are selected for fermentation optimization, based on protein analysis, at the 2 liter scale. The goal is to maintain a high, specific yield (percent rhUG versus total soluble protein) while maximizing biomass. This begins with fermentations to gauge the maximum biomass achievable (maximum OD.sub.600 and wet weight cell paste) in both minimal media, supplemented with glucose, and in rich media at 37 degree. C., followed by a time course of the expression to determine peak uteroglobin production as a function of OD.sub.600 following induction.

Information generated by these two sets of experiments provide the three parameters necessary to initiate preliminary production runs:

1) Use of rich versus minimal media. If comparable results for specific yield and maximum biomass can be obtained, then minimal media is preferable due to materials cost considerations. The time course for a minimal versus a rich media run must also be considered, since doubling time is typically lower in minimal media and the cost of labor on off-shifts is high. Note that for the number of potential applications for rhUG, a large quantity (kilograms per year) may eventually be required.

2) The point at which induction begins is approximately two doubling times prior to entry into stationary phase. The maximal doubling rate is mid-late log phase and is a criterion of

maximizing heterologous protein expression.

3) The expression time course determines the time post-induction at which the culture must be harvested. In rich media, rhUG accumulation is very rapid (up to 50% total protein in 2-4 hours), while in supplemented minimal media, protein accumulation requires several hours (up to 40% of the total protein in 12-18 hours).

The stability of the expression vector is an important issue in the validation of the expression system. It is routinely monitored by comparing cfu's of cells plated from the fermentation inoculum and the harvest, on selective versus non-selective media. Once parameters are established for the initial production runs, stability is initially validated by platings from timepoints in the fermentation as well as by DNA sequencing of the UG gene in plasmids extracted from cells at the beginning and end of the fermentation to verify that no changes have occurred.

Purification of UG; Preparation of Extracts

RhUG will be purified largely according to Mantile et al, 1993; supra and to Miele et al, 1990; supra, with the following modifications. Frozen cell pellets are thawed rapidly and resuspended in approximately 2 mLs of ice cold hypotonic lysis buffer (50 mM NaPO4, pH 10) per gram of wet cell paste. (The use of protease inhibitors (PMSF, leupeptin, and soybean trypsin inhibitor) is necessary in the early stages but may eventually be eliminated). The cell suspension is subjected to 3 freeze-thaw cycle, alternating between a dry ice ethanol bath and a 65 degree. C. for 5-10 minutes and then centrifuged at 30 times g for 20 minutes. Heating the lysate at this point precipitates the bulk of the host proteins and inactivates proteases. The supernatant containing the rhUG is decanted to a clean tube and the pellet is discarded. Except for the heat step, all materials and samples are kept ice cold. RhUG extracts and fractions are examined by

SDS-PAGE (16% Tris-glycine), by protein assay (Pierce BCA kit), and by PLA.sub.2 inhibition activity in vitro according to the manufacturers (EIA, Cayman Chemicals), as needed, to evaluate lysate fractionation steps, as well as to evaluate chromatography, filtration and other purification steps.

Filtration and Chromatography

The purification of rhUG is monitored for recovery of bioactive material after each step in the process. As described above for lysates, SDS-PAGE, protein assay and PLA.sub.2 inhibition assay are used to evaluate steps in the process. Because the end product is to be used in humans, Tris, EDTA and other unnatural components are eliminated from buffers for purification. The introduction of a cost-effective heat step eliminates extra chromatograph steps downstream. Special consideration is also given to non-protein contaminants. The removal of endotoxin and DNA is facilitated by the use of new filtration step.

Clarified bacterial lysates typically have a pH close to neutrality, presumably because the weak buffering strength of the dilute buffer system is overwhelmed by cellular contents once efficient lysis is achieved. The pH of the sample buffer will be dropped to 4.2 with ammonium acetate slowly, with stirring in the cold. RhUG is known to be stable at low pH (Andersson, 1994; supra, Miele, L., et al. High level expression in E. coli of a dimeric, eukaryotic protein with two disulfide bridges under the control of phage T7 promoter. J. Biol. Chem. 265:6427-6435 91990)). The sample is then passed over a Sartobind filter (Sartorius Corp). At low pH, endotoxin and DNA bind with high efficiency to this membrane and are effectively removed. The UG sample is placed on a CM-Sepharose column equilibrated with 25 mM ammonium acetate, pH 4.2. (Column sizes are determined based on protein content in the sample, estimated rhUG content, and sample volume). RhUG binds to the column under these conditions. It is

eluted with a linear gradient of 25 mM ammonium acetate, pH 4.2, and 120 mM ammonium acetate, pH 6.0 (Miele, L., et al, 1990; supra). This process is converted to a step gradient when a reproducible elution profile is obtained. If a second chromatography step is necessary, sample fractions are pooled and concentrated using a YM-2 membrane (Amicon). The sample is then passed over a Sephadex G-75 column equilibrated in 20 mM potassium phosphate, pH 7.2. RhUG fractions are pooled and concentrated again using the YM-2 membrane. The final sample is characterized for activity and concentration. Some aliquots of rhUG are lyophilized and stored at -70.degree. C. with dessicant, others are stored in PBS at 4 degree C.

Molecular Analysis of rhUG

Several methods are used to characterize the final rhUG preparation. The purified dimeric protein is a single band of about 10 kDa on a non-reducing SDS-PAGE gel that collapses to a single 6 kDa band on a reducing SDS-PAGE gel, and is recognized by anti-uteroglobin antibodies in Western blots. Only the dimeric form is active in inhibiting PLA.sub.2 in vitro. PLA.sub.2 activity is measured with an EIA kit (Cayman Chemicals). The activity of each preparation of recombinant human protein is compared to native UG preparation derived from human urine, on an activity versus weight basis (specific activity). Homogeneity is evaluated using analytical HPLC (C-18 reverse phase, 5 micron pore size. TFA:acetonitrile gradient) versus authentic human UG. MALDI-TOF mass spectral analysis is done (M-Scan, Inc.) to verify molecular weight. Amino acid content analysis is done to verify identity (Peptide Technologies, Inc.) N-terminal amino acid sequencing is also done to verify authenticity.

Contaminant Analysis

Testing for E. coli host proteins, endotoxin, and residual bacterial DNA is done. Elimination of host proteins is largely accomplished by the time the protein appears as a single

band on an overloaded silver-stained SDA-PAGE gel, and as a single HPLC peak. However, this is verified in a more sensitive immunoassay (Western dot blot) using polyclonal antibodies generated against E. coli proteins. The levels of DNA and endotoxin contaminants are characterized by the use of a chromogenic LAL assay kit (Becton-Dickenson) and SYBR green dye kit (Molecular Probes), measured in the clarified lysate, before and after the filtration step, and in the final sample. Pure rhUG contains none of these contaminants in detectable quantities, reflecting at least 99% purity.

Stability Testing of rhUG

Studies on the stability of rhUG, as a lyophil, in a frozen liquid at -20.degree. C. and -70.degree. C., and as a liquid suspension kept in the refrigerator and at room temperature are done. The preferred form for cost-effective storage is in liquid room temperature, however, this may not be practical. Purified rhUG preparations continue to be monitored for stability in these forms by testing for PLA.sub.2 inhibition activity over time. Initial time points for resuspended forms were daily for the first week, then twice per week for the first month. The lyophilized samples are stable for at least two years (A.B. Mukherjee, unpublished data), therefore, stability testing was done biweekly for the first month, then monthly for the first year. All rhUG preparations have been stable thus far (A.B. Mukherjee, personal communication).